

Angiogenic inhibition reduces germinal matrix hemorrhage

Praveen Ballabh^{1,2,8}, Hongmin Xu¹, Furong Hu¹, Alex Braun³, Kira Smith⁴, Aracelie Rivera⁴, Nanhong Lou⁵, Zoltan Ungvari⁴, Steven A Goldman^{5,6}, Anna Csiszar⁴ & Maiken Nedergaard⁷

The germinal matrix of premature infants is selectively vulnerable to hemorrhage within the first 48 h of life. To assess the role of vascular immaturity in germinal matrix hemorrhage (GMH), we evaluated germinal matrix angiogenesis in human fetuses and premature infants, as well as in premature rabbit pups, and noted active vessel remodeling in all three. Vascular endothelial growth factor (VEGF), angiopoietin-2 and endothelial cell proliferation were present at consistently higher levels in the germinal matrix relative to the white matter anlagen and cortical mantle. On that basis, we asked whether prenatal treatment with either of two angiogenic inhibitors, the COX-2 inhibitor celecoxib, or the VEGFR2 inhibitor ZD6474, could suppress the incidence of GMH in premature rabbit pups. Celecoxib treatment decreased angiopoietin-2 and VEGF levels as well as germinal matrix endothelial proliferation. Furthermore, treatment with celecoxib or ZD6474 substantially decreased the incidence of GMH. Thus, by suppressing germinal matrix angiogenesis, prenatal celecoxib or ZD6474 treatment may be able to reduce both the incidence and severity of GMH in susceptible premature infants.

In the United States, 1.45% of all live births are premature infants weighing less than 1,500 g. The incidence of germinal matrix hemorrhage–intraventricular hemorrhage (GMH-IVH) in these infants is approximately 20% (12,240 infants per year)^{1,2}. These babies are predisposed to cerebral palsy, hydrocephalus and mental retardation^{3,4}. The germinal matrix, located in the thalamostriate groove beneath the forebrain ventricular wall, is a richly vascularized collection of neural precursor cells—glial precursor cells in particular in the third trimester—which persists until late gestation. The human germinal matrix has a greater density of blood vessels than either the subjacent intermediate zone, the anlagen of the capsular white matter or the developing neocortical mantle. In addition, the germinal matrix has vessels with larger lumina than other brain areas⁵. VEGF treatment of rabbit retina and quail embryonic brain induces the formation of a dense vascular bed, suggesting that VEGF might be involved in the formation of highly vascularized germinal matrix^{6,7}.

VEGF, the predominant angiogenic growth factor of development, triggers endothelial cell proliferation, migration and increased vascular permeability attending the formation of nascent vessels, which essentially consist of immature endothelium with few pericytes and little mature matrix^{8,9}. The angiopoietins ANGPT-1 and ANGPT-2 facilitate VEGF-induced angiogenesis¹⁰. In particular, ANGPT-1 and its endothelial receptor, Tie2, mediate the maturation and stabilization of VEGF-induced vasculature, by promoting the recruitment of smooth muscle cells to the adluminal surface of the newly generated vascular bed¹¹. In contrast, ANGPT-2, a natural antagonist of ANGPT-1, is associated with both initial angiogenesis and capillary destabilization. Mice engineered to overexpress ANGPT-2 resemble both ANGPT-1-deficient and

Tie-2-deficient mice, in that each exhibits dilated and immature blood vessels prone to hemorrhage^{12–15}. An increase in the expression of ANGPT-2 in the presence of VEGF promotes vessel sprouting, whereas in the absence of VEGF, ANGPT-2 signals vessel regression^{9,16,17}.

On this basis, we hypothesized that the germinal matrix might exhibit a high endothelial proliferation index accompanied by increased VEGF and ANGPT-2 levels, potentially leading to instability of the developing vascular bed and predisposing premature neonates to microvascular hemorrhage. Moreover, we postulated that prenatal inhibition of VEGF and ANGPT-2 signaling might decrease the fragility of germinal matrix vasculature and its vulnerability to hemorrhage by suppressing periventricular angiogenesis. We chose celecoxib, an inhibitor of prostaglandin-endoperoxide synthase 2 (PTGS2, colloquially known as COX-2), as a means of suppressing prenatal germinal matrix angiogenesis, because celecoxib has been reported to reverse the hypoxic upregulation of both VEGF and ANGPT-2 in endothelial cells^{18–20} and decrease neovascularization as a result²¹. In addition, celecoxib penetrates both the placental and the blood-brain barriers^{22,23} and has a good safety profile in late pregnancy—indeed it has been used for the treatment of preterm labor²⁴. Although a number of inhibitors of VEGF signaling are available, their systemic toxicity and the lack of experience of their use in pregnant women²⁵, particularly with regard to the small molecule VEGF receptor inhibitors, could limit their use during pregnancy.

In this study, we found that the endothelial proliferation index, and VEGF and ANGPT-2 expression were selectively and substantially increased in the germinal matrix, relative to the cortex and white matter, in both rabbit pups and premature human infants. Prenatal

¹Department of Pediatrics, ²Department of Anatomy & Cell Biology, ³Department of Pathology and ⁴Department of Physiology, New York Medical College-Westchester Medical Center, Valhalla, New York 10595, USA. ⁵Department of Neurosurgery, ⁶Department of Neurology and ⁷Center for Aging and Developmental Biology, University of Rochester, Rochester, New York 15642, USA. ⁸Present address: Regional Neonatal Center, Maria Fareri Children's Hospital, Westchester Medical Center, Valhalla, New York 10595, USA. Correspondence should be addressed to P.B. (pballabh@msn.com).

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celecoxib treatment suppressed each of these metrics in the germinal matrix of preterm rabbit pups, relative to untreated controls, and, most importantly, provided substantial protection against GMH. Similarly, when ZD6474, a small molecule VEGFR2 tyrosine kinase inhibitor, was given to pregnant rabbits, it protected their premature pups against GMH. Together, these studies highlight the propensity of the germinal matrix vascular bed to hemorrhage and suggest mechanism-based strategies for its prevention in the setting of premature delivery.

RESULTS

VEGF is highly expressed by radial glia in the germinal matrix

We evaluated VEGF expression in the human germinal matrix, cortex and white matter by immunohistochemistry, western blot analysis and real-time PCR in 9 human fetuses, 12 premature infants and 3 full-term infants. Immunostaining showed that VEGF was expressed in both blood vessels and glial cells of the human cortex, white matter and germinal matrix (Fig. 1). In the germinal matrix, double labeling

of glial fibrillary acidic protein (GFAP) and VEGF confirmed that VEGF was expressed by radial glia (Supplementary Fig. 1 online). In addition, VEGF also stained glial fibers in the subjacent white matter. Endothelial VEGF colocalized with platelet endothelial cell adhesion molecule-1 (PECAM-1) in endothelial cells in both the cortex and white matter. However, although VEGF immunolabeling was intense in endothelial cells of the cortex and white matter, it was relatively weaker in endothelial cells in the germinal matrix, consistent with previous reports²⁶. Western blotting confirmed that 24 kDa VEGF was significantly more abundant in the germinal matrix, compared to the cortex and white matter, of both human fetuses and premature infants ($P < 0.05$, Fig. 1b). Thus, the increase in VEGF protein level in the germinal matrix seems to be due to its high expression by radial glia, suggesting that VEGF induces angiogenesis in a paracrine fashion²⁷. Accordingly, *VEGFA* mRNA levels were significantly increased in the germinal matrix, compared to the cortex and white matter, of both fetuses and premature infants ($P < 0.05$ both; Fig. 1c).

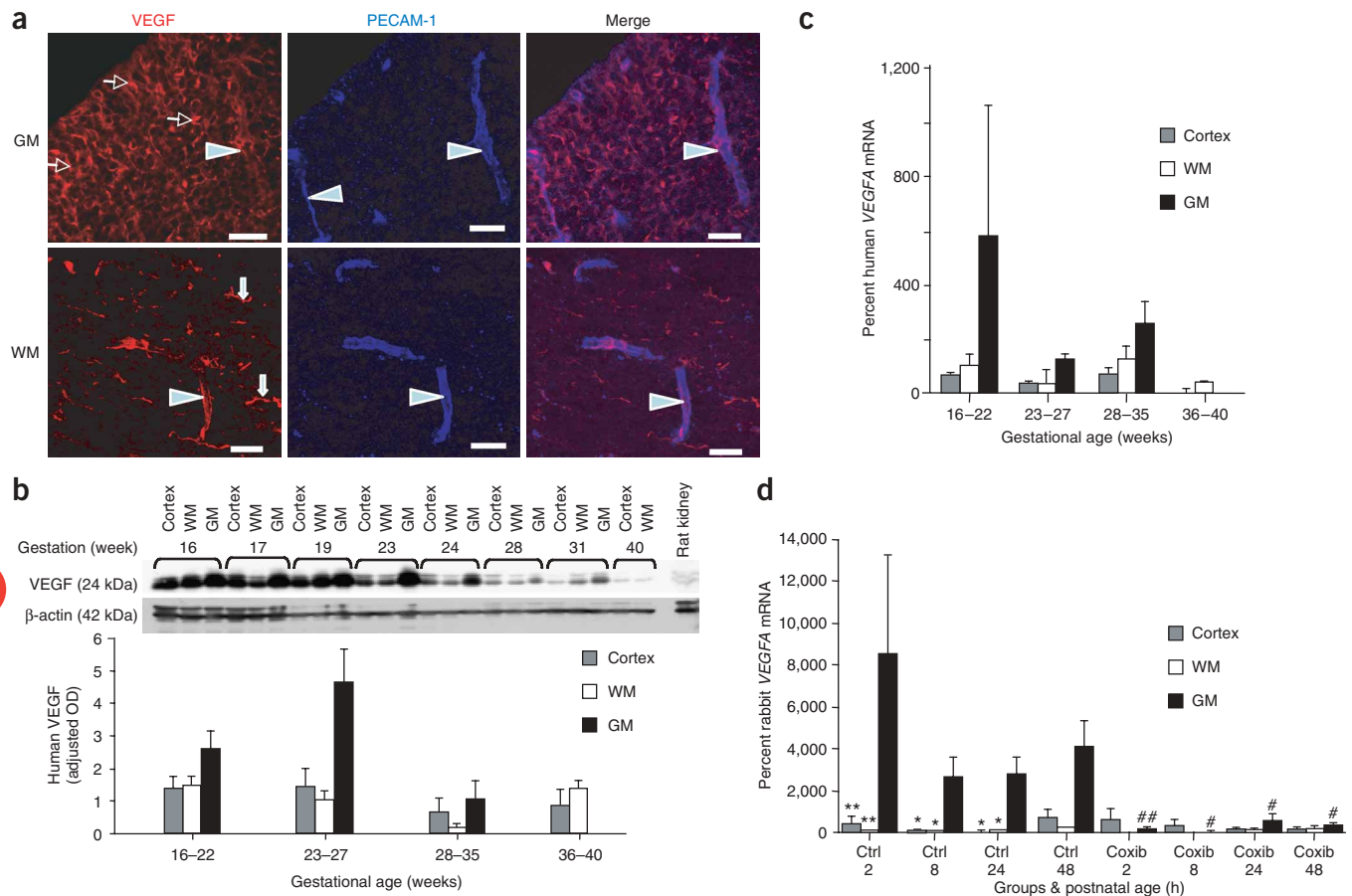


Figure 1 VEGF expression is greater in germinal matrix than in cortex or white matter in premature infants and rabbit pups. **(a)** Representative immunofluorescence of cryosections from germinal matrix (GM) and white matter (WM) of a 20-week premature infant labeled with antibodies to VEGF and an endothelial marker, PECAM-1. In the GM, VEGF stained blood vessels weakly (arrowheads), but stained radial glia (arrows) extensively. In the WM, VEGF stained blood vessels (arrowheads) and also stained glial fibers (block arrows). Scale bars, 20 μ m. **(b)** Western blot analysis of VEGF in post-mortem brain samples from fetuses and premature infants. Rat kidney was used as a positive control. The bar graph shows mean \pm s.e.m. ($n = 24$, fetuses plus premature infants). Values were normalized to β -actin levels. VEGF levels were higher in the GM than in the cortex or WM in both fetuses and premature infants. VEGF expression showed an insignificant downward trend with increasing gestational age ($P = 0.09$). OD, optical density. **(c)** *VEGFA* gene expression assayed by real-time PCR in fetuses and premature infants. Data are mean \pm s.e.m. ($n = 24$, fetuses plus premature infants). *VEGFA* levels were higher in the GM than in the cortex or WM in both fetuses and premature infants. **(d)** Time course of *VEGFA* gene expression, measured by real-time PCR, in control (ctrl) and celecoxib (coxib)-treated rabbit pups at 2, 8, 24 and 48 h of age. Data are mean \pm s.e.m. ($n = 6$ at each time point). Among controls, *VEGFA* levels decreased with advancing gestational age in the GM ($P < 0.04$) but not in the cortex or WM. *VEGFA* levels were significantly higher in the GM than in the cortex or WM at 2, 8 and 24 h postnatal age, but not at 48 h. Celecoxib treatment decreased *VEGFA* levels in the GM. * $P < 0.05$, ** $P < 0.01$ (GM vs. cortex or WM). # $P < 0.05$, ## $P < 0.01$ (control vs. celecoxib-treated pups).

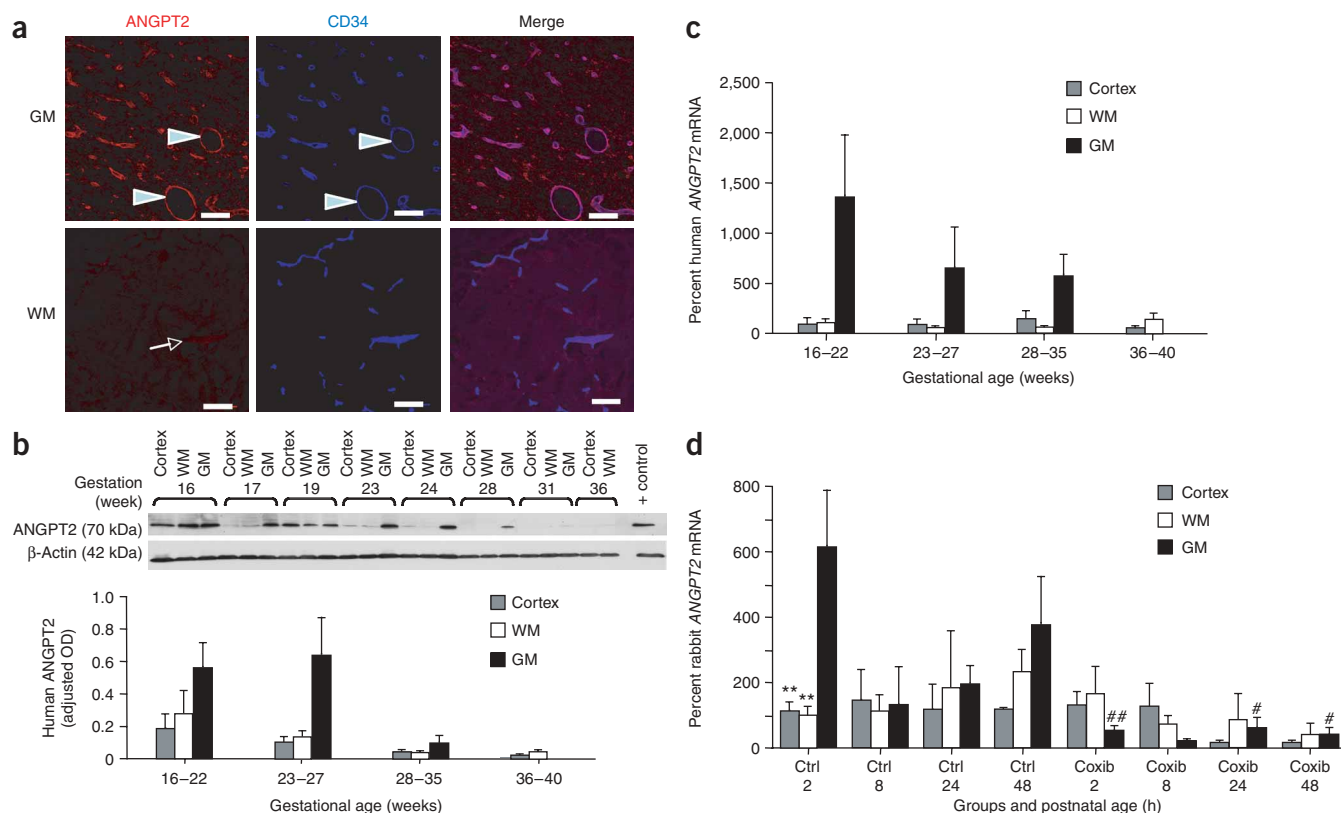


Figure 2 ANGPT-2 levels are greater in the germinal matrix than in the cortex or white matter in both humans and rabbits. **(a)** Cryosections from GM and WM of a 23-week premature infant were double immunostained for ANGPT-2 and CD34. ANGPT-2 was strongly expressed in blood vessels in the GM (arrowheads) and weakly expressed in blood vessels in the WM (arrow). The ANGPT-2 signal colocalized with the endothelial marker CD34. Scale bars, 50 μ m. **(b)** Representative western blot analysis of ANGPT-2 in cortex, WM and GM of eight human subjects. ECV 304 cell lysate (Santa Cruz) was used as positive control. The bar graph shows mean \pm s.e.m. ($n = 24$). The values were normalized to β -actin levels. ANGPT-2 levels were significantly greater in the GM than in the cortex or WM ($P < 0.05$ for all). ANGPT-2 showed a downward trend with increasing gestational age. **(c)** *ANGPT2* gene expression was measured by real-time PCR in fetuses and premature infants. Data are mean \pm s.e.m. ($n = 24$). *ANGPT2* mRNA levels were greater in the GM than in the cortex or WM in both fetuses and premature infants. **(d)** *ANGPT2* mRNA levels assayed by real-time PCR in control and celecoxib-treated pups at 2, 8, 24 and 48 h postnatal age. Data are mean \pm s.e.m. ($n = 6$). In control pups, *ANGPT2* levels were significantly greater in the GM than in the cortex or WM at 2 h postnatal age, but not at 8, 24 or 48 h. Celecoxib suppressed *ANGPT2* in the germinal matrix at 2, 24 and 48 h age. * $P < 0.05$, ** $P < 0.01$ (GM vs. cortex or WM). # $P < 0.05$, ## $P < 0.01$ (control vs. celecoxib-treated pups).

We next evaluated VEGF expression in premature rabbit pups. Like humans, rabbit pups have an abundant germinal matrix, exhibit maximal cerebral expansion perinatally and suffer frequent GMH if delivered prematurely²⁸. Analysis of *VEGFA* mRNA in rabbit pups by real-time PCR at 2, 8, 24 and 48 h of age showed that its level of expression was significantly higher in the germinal matrix compared to the cortex and white matter at 2, 8 and 24 h age ($P < 0.05$ each) but not at 48 h ($P = 0.08$ for germinal matrix vs. cortex; $P = 0.052$ for germinal matrix vs. white matter; **Fig. 1d**). As VEGF plays important roles in neovascularization and hemorrhage⁶ and because VEGF expression is higher in the germinal matrix than in the cortex or white matter (**Fig. 1**), it was important to determine whether down-regulation of VEGF in the germinal matrix would prevent GMH. We selected celecoxib, a PTGS2 and angiogenic inhibitor, because celecoxib reduces endothelial proliferation²⁹, increases apoptosis of angiogenic endothelia²⁹ and has entered phase III trials for cancer treatment³⁰. We treated pregnant rabbits with oral celecoxib (20 mg per kg body weight) twice daily on days 26, 27 and 28 of gestation and delivered pups by cesarean section on day 29. We found that celecoxib suppressed *VEGFA* mRNA expression in the germinal matrix at all time points (that is, 2, 8, 24 and 48 h postnatal age; $P < 0.05$ for all)

but not in the cortex or white matter (**Fig. 1d**). Collectively, these observations show that VEGF expression was increased in both the human and rabbit germinal matrix and that VEGF expression was downregulated as a function of postnatal age and celecoxib treatment in rabbits.

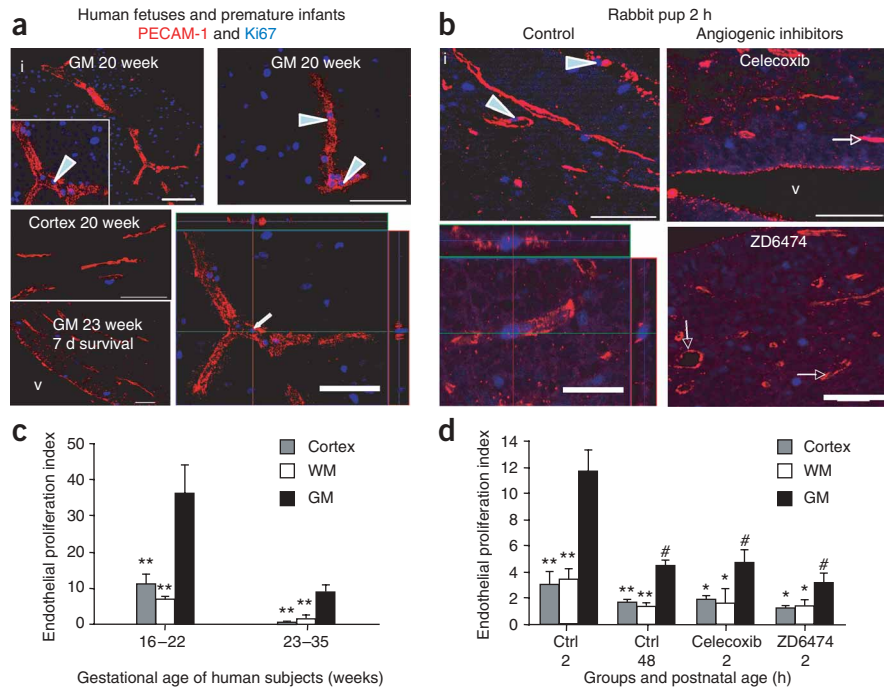
ANGPT-2 is selectively enriched in the germinal matrix

As ANGPT-2 collaborates with VEGF in orchestrating angiogenesis^{16,17}, we next evaluated ANGPT-2 levels in human fetuses and premature infants and in premature rabbit pups. Immunolabeling of coronal brain sections revealed that ANGPT-2 was strongly expressed in the endothelium of the germinal matrix but weakly in the cortex and white matter of fetuses and premature infants (**Fig. 2a**). Western blot analysis also showed that ANGPT-2 protein was more abundant in the germinal matrix than in the cortex or white matter in both fetuses and premature infants (**Fig. 2b**). Similarly, *ANGPT2* mRNA expression was greater in the germinal matrix than in the cortex or white matter in both fetuses (16–22 weeks) and premature infants (23–35 weeks) ($P < 0.05$ each; **Fig. 2c**).

Consistent with these findings, in premature rabbit pups, mRNA expression of *ANGPT2* was significantly higher in the germinal matrix

Figure 3 Endothelial proliferation was higher in GM than in cortex or WM in humans and rabbits. (a) Representative immunofluorescence of cryosections from a 20-week human fetal brain labeled with Ki67 and PECAM-1, showing abundant Ki67-staining nuclei in the GM and a few in the cortex. Note PECAM-1-staining vessels with Ki67 signals indicating endothelial proliferation in the GM (inset and upper right micrograph; arrowheads). Vessels with Ki67 nuclear staining were less frequent in the GM of a 23-week premature infant (7 d of age) than in that of a 20-week fetus. Bottom right micrograph: 20-week fetal brain section from GM shows Ki67 signals within PECAM-1 staining (block arrow). Above and to the right of the image are orthogonal views in *x-z* and *y-z* planes of a composite *z*-stack of a series of confocal images taken 0.4 μm apart. V, ventricle. Scale bars, 20 μm . (b) Cryosections from celecoxib- and ZD6474-treated pups and controls of age 2 h were immunolabeled with Ki67 and PECAM-1. In a control pup, Ki67 signals overlapping PECAM staining indicated proliferating endothelium in the GM (arrowheads). In pups treated prenatally with celecoxib or ZD6474, PECAM-1-staining blood vessels lacked Ki67 signals (arrows). Shown in the bottom left micrograph, above and

to the right of the image, are orthogonal views in *x-z* and *y-z* planes of a composite *z*-stack of a series of confocal images taken 0.5 μm apart; the images depict Ki67-staining nuclei within PECAM-1 staining cells in the GM of a control pup. V, ventricle. Scale bars, 20 μm . (c) Endothelial proliferation in human fetuses ($n = 6$) and premature infants ($n = 6$) is shown (mean \pm s.e.m.). Endothelial proliferation index was significantly greater in the GM than in the cortex or WM both in fetuses and premature infants, and was significantly greater in fetuses than in premature infants. * $P < 0.05$, ** $P < 0.01$. (d) Endothelial proliferation in four groups of pups as indicated ($n = 6$ each) is shown (mean \pm s.e.m.). Endothelial proliferation was significantly higher in the GM than in the cortex or WM in all groups; * $P < 0.05$, ** $P < 0.01$ (GM vs. Cortex or WM). Endothelial proliferation was lower at 48 h than at 2 h postnatal age among control pups ($P < 0.05$). Celecoxib or ZD6474 treatment reduced endothelial proliferation in the GM at 2 h age. # $P < 0.05$ (control 2 h vs. control 48 h; control 2 h vs. celecoxib-treated; and control 2 h vs. ZD6474-treated pups).



than in the cortex or white matter at 2 h of age ($P < 0.05$ each) but not at 8, 24 or 48 h of age (Fig. 2d). Notably, celecoxib suppressed *ANGPT2* gene expression in the germinal matrix at 2, 24 and 48 h of age ($P < 0.05$ each) but not in the cortex or white matter. Hence, like VEGF, *ANGPT-2* expression was greater in the germinal matrix than in the cortex or white matter in humans and rabbits, and prenatal celecoxib treatment inhibited *ANGPT-2* in the germinal matrix of rabbits.

VEGFR and Tie-2 levels are comparable among brain regions

As VEGF expression was increased in the germinal matrix compared to the cortex and white matter, it was important to evaluate the expression of its receptors. We found that VEGFR1 (gene symbol: *FLT1*) and VEGFR2 (gene symbol: *KDR*) protein and mRNA expression in human fetuses, premature infants and rabbit pups was not substantially different among cortex, white matter and germinal matrix, and did not change with postnatal age (Supplementary Figs. 2 and 3 online).

Tie-2, a receptor of *ANGPT-1*, mediates endothelial cell stability and is a critical regulator of angiogenesis¹¹. Tie-2 protein and *TEK* (also known as *TIE2*) mRNA expression in fetuses and premature infants were not substantially different among cortex, white matter and germinal matrix, and did not change with advancing gestational age. Likewise, mRNA expression in rabbit pups was similar in the germinal matrix, cortex and white matter (Supplementary Fig. 4 online). Hence, Tie-2 expression, like that of its ligand *ANGPT-1*, seems to mature early in gestation in all three areas of the brain. These findings are consistent with the previous observation

that Tie-2 is detectable in the mouse brain as early as embryonic day (E) 12.5 (ref. 31).

ANGPT-1 is more abundant in fetuses than in premature infants

ANGPT-1 plays a crucial role in endothelial cell differentiation, recruitment of pericytes and vascular maturation^{9,11}. Therefore, we assessed *ANGPT-1* expression in perinatal human and rabbit brain samples. Immunolabeling of coronal brain sections from human fetuses and premature infants showed that *ANGPT-1* was expressed in blood vessels, astrocyte cell bodies and processes as well as in the radial glia. Western blot analysis revealed that *ANGPT-1* protein levels were comparable among cortex, white matter and germinal matrix in fetuses, premature and mature infants. However, in all brain regions, *ANGPT-1* protein level was greater in fetuses than in premature infants ($P < 0.05$). Real-time PCR revealed that there was no substantial difference in mRNA expression of *ANGPT-1* among the three areas. However, unlike protein expression, *ANGPT-1* mRNA was not greater in fetuses compared to premature infants. These data suggest that *ANGPT-1* protein may be subject to increased turnover in the fetal brain (Supplementary Fig. 4).

Evaluation of mRNA in premature rabbit pups showed no statistical difference among *ANGPT1* mRNA levels in the cortex, white matter and germinal matrix, or between levels at 2 and 48 h postnatal age ($P > 0.15$ for all; Supplementary Fig. 5 online). Greater *ANGPT-1* expression in fetuses than in premature infants and its appearance as early as 16 weeks indicate that *ANGPT-1* is expressed early in gestation in different brain areas.

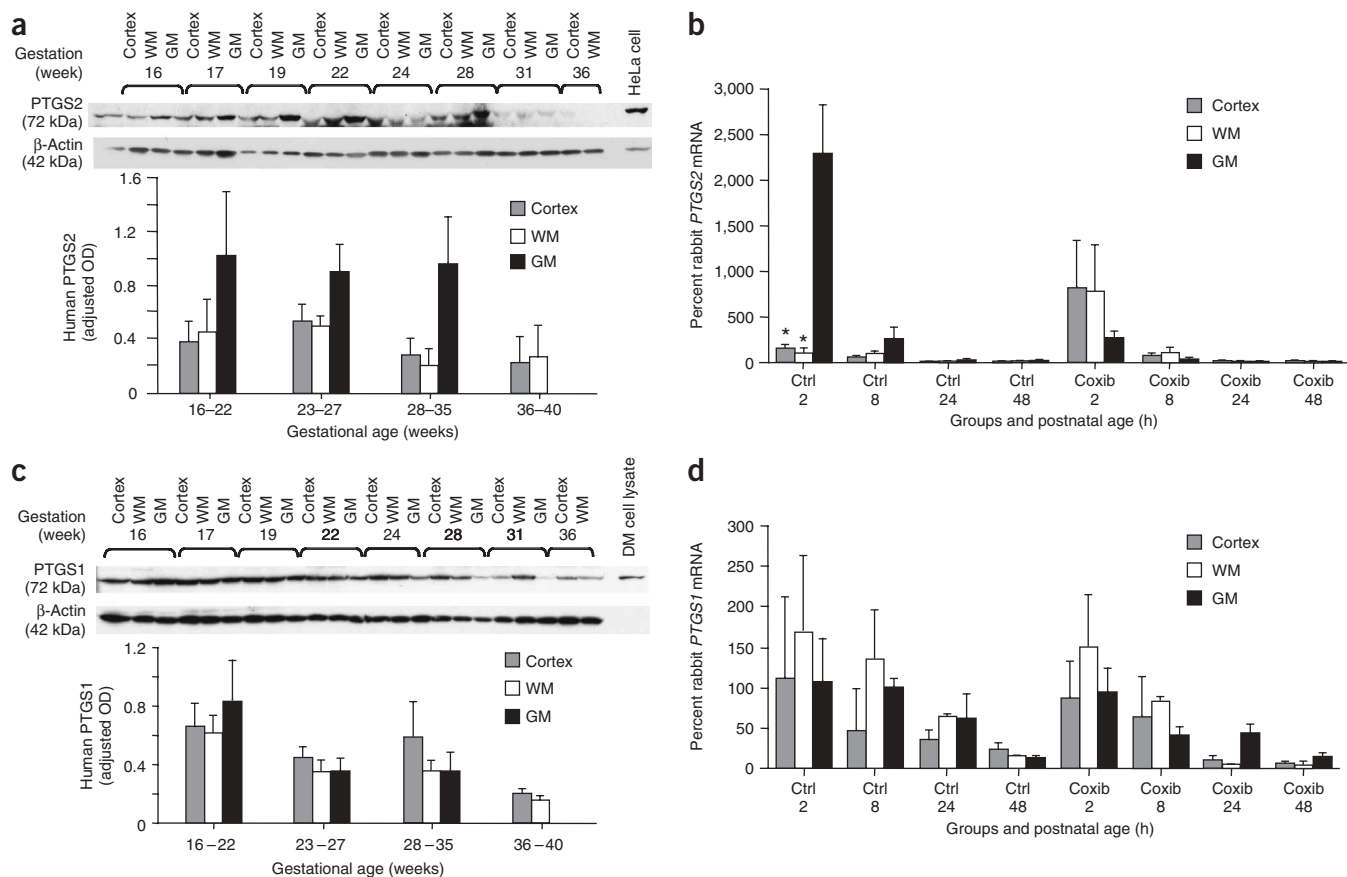


Figure 4 PTGS2 levels, but not PTGS1 levels, are higher in the germinal matrix than in the cortex or white matter. **(a)** Representative western blot analysis of PTGS2 in the cortex, WM and GM of eight human subjects. HeLa cell lysate was used as a positive control. The bar graph shows mean \pm s.e.m. ($n = 24$). PTGS2 levels were normalized to β -actin levels. PTGS2 levels were greater in the GM than in the cortex or WM in each age group ($P < 0.05$) and did not change with gestational age. **(b)** PTGS2 gene expression measured at 2, 8, 24 and 48 h age in control and celecoxib-treated pups by real-time PCR. Data are mean \pm s.e.m. ($n = 6$ at each time point). In controls, PTGS2 levels diminished with increasing postnatal age in the GM but not in the cortex or WM. PTGS2 expression was significantly higher in the GM than in the cortex or WM at 2 h of age, but not at 8, 24 or 48 h. Celecoxib suppressed PTGS2 expression in the GM at 2 h, but not at other time points. $*P < 0.05$. **(c)** Representative western blot analysis of PTGS1 in the cortex, WM and GM of eight human subjects. COL0320 DM cell lysate (Santa Cruz) was used as a positive control. The bar graph shows mean \pm s.e.m. ($n = 24$). PTGS1 levels were normalized to β -actin levels. PTGS1 expression was similar in the cortex, WM and GM, and decreased significantly with increasing gestational age in all three areas ($P < 0.05$ each). **(d)** Time course of PTGS1 gene expression, measured by real-time PCR, in control (ctrl) and celecoxib (coxib)-treated rabbit pups at 2, 8, 24 and 48 h of age. Data are mean \pm s.e.m. ($n = 4$ at each time point). Among untreated pups, PTGS1 gene expression showed a downward trend with increasing postnatal age. PTGS1 gene expression was similar in the cortex, WM and GM, and was not affected by celecoxib treatment.

Endothelial proliferation in germinal matrix

As the levels of VEGF and ANGPT-2, key inducers of angiogenesis^{9,10}, were greater in the germinal matrix than in the other brain areas, we next evaluated endothelial proliferation in the germinal matrix vasculature and the effect of prenatal treatment with celecoxib or ZD6474 on endothelial proliferation in the germinal matrix of rabbit pups. We chose Ki67 to assess endothelial proliferation because it is a marker for proliferating cells and stains cells in late G1, S, G2 and M phases of the cell cycle³². The endothelial cell proliferative index was calculated as the percentage of all Ki67-positive nuclei within PECAM-1-positive endothelial cells. To verify Ki67 immunoreactivity embedded within PECAM-1 staining, we evaluated x - z and y - z (orthogonal views) reconstructions of stacks of confocal images (Fig. 3a,b).

Double immunostaining of human brain sections with Ki67 and PECAM-1 showed that the endothelial proliferation index was significantly greater in the germinal matrix than in the cortex or white matter both in fetuses and premature infants ($P < 0.01$ each,

Fig. 3a,c). In addition, endothelial proliferation was significantly greater in the germinal matrix of fetuses than in that of premature infants ($P < 0.05$). As the delivered fetuses had a gestational age of 18.6 ± 0.8 weeks (mean \pm s.e.m.) and succumbed shortly after birth, whereas premature infants had a gestational age of 26.5 ± 0.9 weeks and a mean postnatal age of 7.4 ± 2.1 d, the difference in endothelial proliferation between fetuses and premature infants suggests that angiogenesis decreases shortly after birth. This decrease may be a result of decreased VEGF and ANGPT-2 expression resulting from increased arterial oxygenation³³.

We obtained similar findings in rabbit pups (Fig. 3b,d). The endothelial proliferation index was significantly greater in the germinal matrix than in the cortex or white matter in both control pups ($P < 0.01$) and in pups exposed to celecoxib or ZD6474 at 2 h postnatal age ($P < 0.05$ each). Furthermore, endothelial proliferation in rabbit germinal matrix was lower at 48 h than at 2 h postnatal age in untreated controls ($P < 0.05$), and, notably, endothelial proliferation in the germinal matrix of 2-h-old pups treated with celecoxib and

Table 1 Incidence of glycerol-induced GMH-IVH in premature rabbits as a function of celecoxib and ZD6474 treatment

Group #	Treatment	<i>n</i>	Weight (g)	Heart rate (per min)	Subarachnoid hemorrhage	No GMH-IVH	Mild GMH-IVH	Moderate GMH-IVH	Severe GMH-IVH
1	Glycerol i.p. 2 h age	20	39 ± 1.76	218 ± 11.2	18 (90)	0 (0)	2 (10)	8 (40)	10 (50)
2	Saline i.p. 2 h age	20	34.8 ± 1.7	205 ± 9.9	0 (0)	16 (80)	3 (15)	1 (5)	0 (0)
3	Glycerol i.p. 48 h age	20	37.4 ± 1.8	217 ± 12	9 (45)	9 (45)	6 (30)	5 (25)	0 (0)
4	Saline i.p. 48 h age	20	36.5 ± 2.1	226 ± 10.1	9(0)	19 (95)	1 (5)	0 (0)	0 (0)
5	Glycerol i.p. 2 h age Prenatal celecoxib	20	37.9 ± 3.1	202 ± 12.9	19 (95)	4 (20)	7 (35)	7(35)	2 (10)
6	Saline i.p. 2 h age Prenatal celecoxib	19	38.6 ± 2.5	221 ± 13.1	1 (5)	18 (95)	1 (5)	0 (0)	0 (0)
7	Glycerol i.p. 2 h age Prenatal ZD6474	26	38.4 ± 1.6	205 ± 12	20 (77)	12 (46)	7 (27)	4 (15)	3 (12)
8	Saline i.p. 2 h age Prenatal ZD6474	26	36.5 ± 2.1	209 ± 11	0 (0)	25 (96)	1 (4)	0 (0)	0 (0)

Numbers in parentheses indicate percentages. Comparison of heart rate and weight among the groups was not significant ($P = 0.14$). Comparison of no gross IVH (no hemorrhage + mild hemorrhage) versus gross IVH (moderate + severe hemorrhage) among groups 1, 3 and 5 was significant ($P < 0.0013$, χ^2 test). Gross IVH decreased as a function of postnatal age (group 1 versus group 3; $P < 0.001$) and in response to celecoxib treatment (group 1 versus group 5; $P < 0.01$) (Fisher exact test, level of significance on Bonferroni correction was $P < 0.017$). Gross IVH was decreased by ZD6474 treatment (group 1 versus group 7, $P < 0.001$, Fisher exact test). Celecoxib and ZD6464 protected equally against IVH (group 5 versus group 7, $P > 0.05$, Fisher exact test).

ZD6474 was lower than that in control pups of the same age ($P < 0.01$). In conclusion, there was a high level of endothelial proliferation in the germinal matrix vasculature of both humans and rabbits, which was suppressed in rabbit pups by prenatal administration of celecoxib or ZD6474.

PTGS2 and PTGS1 expression

PTGS2 promotes angiogenesis by upregulating VEGF and ANGPT-2 (refs. 19, 20), and our data indicated that celecoxib, a PTGS2 inhibitor, suppressed angiogenesis. It was therefore important to assess PTGS2 expression in human and rabbit germinal matrix, cortex and white matter. Western blot analysis showed that PTGS2 levels were greater in the germinal matrix than in the cortex or white matter of both fetuses and premature infants ($P < 0.05$ both; **Fig. 4a**). Accordingly, the expression of PTGS2 mRNA in premature rabbit pups was significantly greater in the germinal matrix than in the cortex or white matter at 2 h ($P < 0.01$, each) but not at 8, 24 or 48 h postnatal age. PTGS2 mRNA levels decreased as a function of postnatal age ($P < 0.01$) and were almost undetectable at 24 and 48 h age in all areas. Notably, celecoxib significantly suppressed PTGS2 gene expression in the germinal matrix at 2 h of age ($P < 0.01$) but did not further reduce PTGS2 levels at the subsequent time points (**Fig. 4b**). Downregulation of PTGS2 by celecoxib has been described previously^{34,35} and was ascribed to prostaglandin E2 (PGE2)-mediated induction of PTGS2 via activation of the Ras-MAPK (mitogen-activated protein kinase) pathway¹⁸. We therefore compared forebrain PGE2 levels of pups exposed to celecoxib to those of untreated controls (5 pups each). Using ELISA of tissue homogenates, PGE2 levels were significantly lower in pups treated with celecoxib than in untreated controls (0.63 ± 0.13 versus 5.6 ± 1.9 ng/ml; $P < 0.001$). Thus, both rabbit and human germinal matrix had higher PTGS2 levels than surrounding brain areas, and PTGS2 levels fell with age or with prenatal celecoxib exposure in premature rabbit pups.

Because celecoxib might also inhibit PTGS1 (also known as COX-1), we asked whether PTGS1 was upregulated in the human and rabbit germinal matrix and whether celecoxib treatment might affect PTGS1. Western blot analysis revealed that PTGS1 protein levels were similar in the germinal matrix, cortex and white matter, in both fetuses and premature infants (**Fig. 4c**). In addition, PTGS1 levels decreased with advancing gestational age in all three regions ($P < 0.05$ each). Similarly, PTGS1 mRNA gene expression in rabbits was comparable among cortex, white matter and germinal matrix at 2, 8, 24 and 48 h of age, and showed a trend for decreasing levels with

postnatal age. Notably, celecoxib did not affect PTGS1 expression. Thus the effect of celecoxib seems principally mediated by the inhibition of PTGS2, but not PTGS1, in the germinal matrix.

Celecoxib attenuates IVH in premature rabbit pups

As celecoxib suppressed VEGF, ANGPT-2 and PTGS2 levels, we asked whether prenatal celecoxib decreased the propensity of germinal matrix to hemorrhage in the premature rabbit pups. To this end, we used a premature rabbit pup model in which IVH was provoked by producing intracranial hypotension with intraperitoneal glycerol¹³⁶. Timed pregnant rabbits ($n = 17$) were alternately assigned to receive either oral celecoxib (20 mg/kg) twice daily on days 26, 27 and 28 of gestation, or no treatment. We did not observe any adverse effects in either the dam or in premature newborns treated with prenatal celecoxib. We performed cesarean section to deliver pups on day 29 of gestation. The control pups (not exposed to celecoxib) were sequentially assigned to receive glycerol at 2 h postnatal age, glycerol at 48 h, saline at 2 h or saline at 48 h. In contrast, the pups treated with prenatal celecoxib were assigned to receive either glycerol or saline at 2 h postnatal age. In the prenatal celecoxib treatment group, we did not administer saline or glycerol at 48 h of age as the incidence of IVH was very low in the control pups at this age (**Table 1**). All the premature pups were killed 24 h after glycerol or saline administration to evaluate the incidence of GMH-IVH. IVH was classified by a modification of Papile's grading³⁷ as either mild (microscopic hemorrhage detected on H&E-stained brain sections), moderate (gross hemorrhage into lateral ventricles without significant ventricular enlargement) or severe (significant ventricular enlargement and/or intraparenchymal hemorrhage) (**Fig. 5**). Mild, moderate and severe IVH developed in 10%, 40% and 50%, respectively, of control pups receiving glycerol at 2 h of age, and in 30%, 25% and 0%, respectively, of control pups that received glycerol at 48 h (**Table 1**). Notably, at 48 h postnatal age, the pups were completely protected against severe IVH and the overall proportion of pups developing IVH fell to 55% compared to 100% at 2 h age ($P < 0.001$). Thus, just as in humans, postnatal maturation protected the rabbit pups against IVH.

In pups exposed to prenatal celecoxib, mild, moderate and severe IVH developed in 35%, 35% and 10% of cases after glycerol was administered at 2 h of age. As microscopic (mild) hemorrhage is usually not associated with clinical complications, we combined no hemorrhage cases with mild cases (no gross IVH) and combined moderate cases with severe cases (gross IVH). Three groups were compared: untreated controls at 2 and 48 h postnatal age and pups

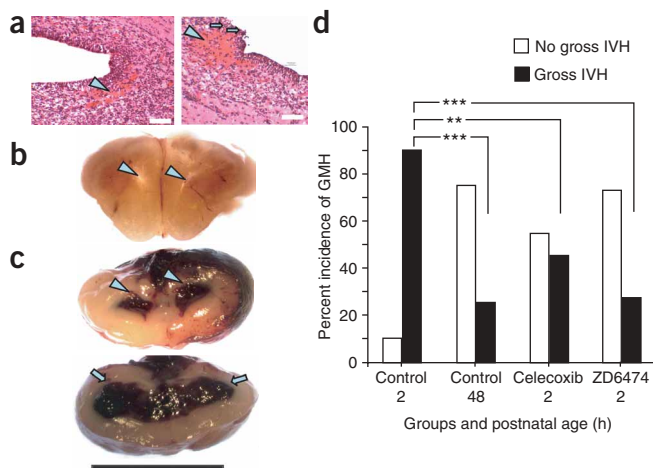


Figure 5 Germinal matrix hemorrhage in rabbit pups. **(a)** H&E-stained coronal brain section shows microscopic hemorrhage (arrowhead) in the GM of a celecoxib-treated pup (left). Note a microscopic hemorrhage (arrowhead) with an apparent destruction of GM margin (block arrows) in a control pup with gross hemorrhage (right). Scale bar, 100 μ m. **(b)** Coronal brain section through frontal lobe of a premature rabbit pup brain shows a normal slit-like ventricle (arrowheads), indicating no IVH. This pup received prenatal celecoxib and i.p. glycerol. **(c)** Coronal brain sections through the frontal lobe of two untreated controls, showing IVH after i.p. glycerol. Top, moderate IVH (arrowheads) without substantial ventricular dilatation. Bottom, severe IVH (block arrows) with dilatation and fusion of the two ventricles into a single chamber. Scale bar **(b and c)**, 1 cm. **(d)** Pups treated with celecoxib or ZD6474 had a lower incidence of GMH than untreated controls. Data are presented as percent pups with either gross IVH or no gross IVH among four subsets of pups: (i) control pups receiving glycerol at 2 h ($n = 20$), (ii) control pups receiving glycerol at 48 h ($n = 20$), (iii) celecoxib-treated pups receiving glycerol at 2 h ($n = 20$) and (iv) ZD6474-treated pups receiving glycerol at 2 h ($n = 26$). Fisher exact test was used for comparisons. The level of significance after Bonferroni correction was < 0.017 (untreated controls at 2 h versus celecoxib-treated pups). $**P < 0.01$, $***P < 0.001$.

treated with celecoxib at 2 h postnatal age (Fig. 5d). Because the three-group comparison was significant ($P < 0.0001$, χ^2 test), we made further pairwise comparisons between groups. We found that gross IVH was significantly lower in pups treated with celecoxib than in untreated controls of the same postnatal age ($P < 0.01$, Fisher exact test; level of significance with Bonferroni correction is $P < 0.017$). In addition, severe IVH was less in celecoxib-treated pups than in controls ($P < 0.01$). Hence, celecoxib decreased the severity and the incidence of GMH in premature pups.

As celecoxib might influence the hemodynamics of cerebral circulation³⁸, we monitored mean arterial blood pressure, intracranial pressure and cerebral blood flow in celecoxib-treated as well as control pups (Supplementary Fig. 6 online). All three metrics were comparable between celecoxib-treated and control pups, so that the protective effect of celecoxib on the germinal matrix, in particular its suppression of GMH, seems to be independent of effects of celecoxib on regional blood flow. In addition, glycerol had no demonstrable effect on angiogenesis or VEGF: intraperitoneal glycerol did not influence endothelial proliferation or induce VEGF or ANGPT-2 expression in the germinal matrix of pups (Supplementary Fig. 7 online).

Prenatal ZD6474 minimizes IVH in premature rabbit pups

To further assess the mechanistic link between VEGF and GMH, we treated a separate set of six pregnant rabbits with the small molecule VEGF inhibitor ZD6474 (20 mg/kg, administered orally once a day) on days 26, 27 and 28 of gestation. ZD6474 suppressed phosphorylation of VEGFR2 and ERK1/2 in the germinal matrix of rabbit pups (Supplementary Fig. 8 online). There were no adverse effects in the dam treated with ZD6474, nor stillbirths in pups exposed to prenatal ZD6474. Pups, delivered by Cesarean section at 29 d, were alternately treated with either glycerol or saline at 2 h of age. Comparison of the incidence of gross IVH in ZD6474-treated pups and controls (Fig. 5d and Table 1) showed that ZD6474 protected the pups against glycerol-induced IVH ($P < 0.001$, Fisher exact test). We also compared the efficacies of celecoxib and ZD6474 in preventing glycerol-induced IVH, and found no difference in gross IVH versus no gross IVH or in no IVH versus IVH (gross + microscopic) between the two treatments.

DISCUSSION

As premature infants are rescued at progressively earlier gestational ages, there is an increase in both the frequency and severity of GMH

(refs. 1,39). GMH and its attendant complications, which include seizure disorders, cerebral palsy and impaired cognitive development, have thus developed as important public health concerns³. In this study, we explored the mechanistic and therapeutic implications of the observation that 51% of all GMH incidents are detected in the first 8 h of life, and 90% in the first 48 h, regardless of gestational age^{40,41}. This suggests that the germinal matrix vasculature has an inherent fragility in preterm infants that resolves rapidly after birth but predisposes the infant to potentially catastrophic hemorrhages until such vascular stabilization is achieved. In the present study we observed that angiogenesis was preferentially active in the human and rabbit germinal matrix, relative to other brain parenchymal compartments, and that regions of high endothelial proliferative activity corresponded to those most susceptible to matrix hemorrhage. We then found that prenatal celecoxib treatment markedly decreased the endothelial proliferation index and the levels of ANGPT-2 and VEGF in the germinal matrix of premature rabbit pups compared to untreated controls. Furthermore, rabbit pups exposed to celecoxib or ZD6474 were less vulnerable to GMH than were untreated controls. Hence, enhanced angiogenesis in the germinal matrix attended by high VEGF and ANGPT-2 seems to be responsible for its propensity to hemorrhage. Accordingly, we found that the prenatal inhibition of angiogenesis by celecoxib or ZD6474 treatment reduced the incidence of GMH, just as did postnatal maturation.

A number of previous studies have indicated that angiogenesis induced by elevated levels of VEGF and ANGPT-2 is associated with vascular fragility and increased propensity to hemorrhage. First, injection of a combination of VEGF and ANGPT-2 into the optic tectum of a 4-d-old quail embryo caused retro-orbital or intraventricular hemorrhage and brain blood vessels appeared enlarged and dysmorphic⁶. Second, the presence of VEGF and ANGPT-2 causes destabilization of blood vessels with formation of new sprouts and the elaboration of new vascular beds, by a series of well-coordinated mechanisms that include vasodilatation, increased vascular leakage, endothelial proliferation and migration, and degradation of basement membrane by activation of urokinase-type plasminogen activator^{9,15–17}. Third, mice overexpressing ANGPT-2 exhibit dilated and immature blood vessels that are prone to hemorrhage¹⁴. Fourth, upregulation of ANGPT-2 causes detachment of perivascular pericytes, which in turn leads to the development of microaneurysms and hemorrhages in the diabetic retina¹³. Thus, ANGPT-2 seems to collaborate with VEGF to increase the propensity of the germinal matrix vasculature to hemorrhage.

VEGFA, *ANGPT2* and *PTGS2* gene expression as well as endothelial proliferation were enhanced in the germinal matrix compared to the cortex or white matter, and were decreased as a function of postnatal age or by celecoxib treatment. These observations suggest a mechanistic link between *PTGS2*, *VEGFA* and *ANGPT2* gene expression and increased angiogenesis. A number of researchers have noted a relationship between *PTGS2* and angiogenesis^{42,43}. The role of *PTGS2*, which catalyzes the formation of PGE2 from arachidonic acid, is of particular importance in this regard. PGE2 induces the expression of *VEGFA* in tumor cells as well as in retinal endothelial cells, and this induction is mediated by hypoxia-inducible factor (HIF-1 α)^{44,45}. PGE2 is also a transcriptional activator of *ANGPT2* in endothelial cells through a HIF-1 α -independent mechanism¹⁹. Thus, *PTGS2* and its product PGE2 may regulate germinal matrix VEGF through HIF-1 α and may also directly regulate germinal matrix *ANGPT2*.

Celecoxib is able to penetrate the placental and blood-brain barriers and can thus penetrate the fetal germinal matrix if administered to a pregnant mother^{22,23}, and celecoxib is safe for use in pregnant women for 3–5 days in late gestation²⁴. Our finding that prenatal administration of celecoxib decreased GMH in rabbit pups leads us to propose that celecoxib be investigated in a clinical trial in which pregnant mothers in preterm labor would be treated to prevent GMH in premature infants. We also found that ZD6474, a small molecule VEGFR2 and epidermal growth factor receptor inhibitor, effectively protected premature pups against glycerol-induced GMH, consistent with the notion that inhibition of angiogenesis decreases the fragility of germinal matrix vasculature and its propensity to hemorrhage. We did not notice any apparent adverse effect of ZD6474 either on the dam or on newborn pups, but it is uncertain whether ZD6474 is safe for use in pregnant women. Of the two medications, celecoxib, which has an additional benefit of suppressing preterm labor in pregnant women^{24,46}, would seem to be the more attractive agent for clinical testing. If celecoxib proves safe and effective in preventing GMH in the clinical setting, then its use could potentially have a major impact on the survival, neurological integrity and developmental outcome of premature infants.

METHODS

Human subjects. The Institutional Review Board at New York Medical College and Westchester Medical Center approved the use of human autopsy materials for this study. The study material included brain tissue sampled from infants of 23–40 weeks gestational age and from spontaneous abortuses of 16–22 weeks gestational age. Samples were obtained less than 18 h post mortem for the premature infants and less than 8 h for the fetuses. We excluded infants with major congenital anomalies, chromosomal defects, culture-proven sepsis, meningitis or hypoxic-ischemic encephalopathy and infants receiving extracorporeal membrane oxygenator treatment. We obtained relevant clinical information from the pathologist in such a manner that subjects could not be identified directly or through identifiers linked to subjects. We included 9 human fetuses, 12 premature infants and 3 full-term infants for immunohistochemistry, western blot analysis and real-time PCR for all markers. However, we used only 6 human fetuses and 6 premature infants for the evaluation of endothelial proliferation index.

Human tissue collection and processing. The wall of the fetal cerebral hemisphere consists of the ventricular zone, subventricular zone, intermediate zone, cortical plate and marginal zone as described by the Boulder Committee⁴⁷. In the present study, for the sake of simplicity and uniformity of presentation, ‘white matter’ refers to intermediate-zone embryonic white matter and ‘cortex’ refers to the cortical plate. Brain samples were processed as described previously⁴⁸. Briefly, we obtained 1- to 2-mm-thick coronal blocks by sectioning through frontal cortex (cortical plate), frontal white matter (embryonic intermediate layer) and germinal matrix in

the region of the thalamostriate groove. The samples were fixed in 4% paraformaldehyde in phosphate buffer saline (PBS; 0.01 M, pH 7.4) for 18 h, and then were cryoprotected by immersion into 20% sucrose in PBS buffer for 24 h followed by 30% sucrose for the next 24 h. We froze tissues after embedding them into optimum cutting temperature compound (Sakura). Frozen coronal blocks were cut into 20- μ m sections using cryostat and saved at -80° C until use.

Immunohistochemistry, western blot analysis and quantitative real-time PCR. We performed these techniques as described in **Supplementary Methods** online.

Animal experiments. The Institutional Animal Care and Use Committee of New York Medical College approved the animal protocol. We obtained 17 timed pregnant New Zealand rabbits from Charles River Laboratories. Rabbits were alternately assigned to receive either oral celecoxib ($n = 8$) or no treatment ($n = 9$). We performed cesarean section at day 29 of gestational age (full-term gestation for rabbit is 31–32 d). The pups born to pregnant rabbits exposed to celecoxib were alternately assigned to receive either glycerol or saline at 2 h postnatal age, whereas pups not exposed to celecoxib (control) were sequentially assigned to receive (i) 6.5 g/kg of 50% glycerol intraperitoneally (i.p.) at 2 h, (ii) 13 ml/kg saline i.p. (control) at 2 h, (iii) 6.5 g/kg of 50% glycerol i.p. at 48 h and (iv) 13 ml/kg saline i.p. (control) at 48 h postnatal age. Live born and healthy looking pups were included in the study. Pups with postnatal complications including aspiration of formula, cardiac arrest requiring resuscitation or any apparent congenital defect were excluded from the study. We dried pups immediately after cesarean section and suctioned their mouths. They were kept warm in an infant incubator, which was maintained at a temperature of 35 $^{\circ}$ C. We fed them 1 ml KMR (PETAG Inc.) at 4 h of age and then 2 ml every 12 h (100 ml/kg/d) using a 3.5 French feeding tube.

For celecoxib treatment, pregnant rabbits received 20 mg/kg celecoxib (Celebrex, Pfizer) twice daily on days 26, 27 and 28 of gestation. Celecoxib was administered orally by mixing the content of the capsule into water. We used a feeding catheter to deliver celecoxib into the oral cavity of the dam.

For ZD6474 treatment, we performed a separate experiment in which we treated six consecutive pregnant rabbits with oral ZD6474 (AstraZeneca) once daily (20 mg/kg) on days 26, 27 and 28 of gestation.

Induction of GMH in premature rabbit pups. We performed cesarean section on day 29 of gestational age. We induced intracranial hypotension by injecting 6.5 g/kg of 50% glycerol (about 0.5–0.7 ml) i.p. in premature pups at about 2–3 h of postnatal age. Pups were killed at 24 h after injection of glycerol or saline. Brains were sectioned into 2-mm blocks starting from the cranial end of the cerebral hemisphere, and were examined for gross ventricular hemorrhage. Slices from the brain without gross hemorrhage were evaluated for microscopic hemorrhages. Brain slices without gross hemorrhage were fixed in 4% paraformaldehyde in phosphate buffer saline and cryoprotected by immersing into sucrose as described above for human tissue processing. Frozen coronal blocks were cut into 10- μ m sections using a cryostat. Every 20th section of the 2nd and 3rd slices obtained from the cranial end of the cerebral hemisphere was stained with hematoxylin and eosin (H&E) stain and evaluated for microscopic hemorrhage. IVH was classified based on a modification of Papile’s grading³⁷: (i) mild, no gross hemorrhage and hemorrhage detected on microscopy of H&E-stained brain sections; (ii) moderate, gross hemorrhage into lateral ventricles without significant ventricular enlargement; and (iii) severe IVH with significant ventricular enlargement and/or intraparenchymal hemorrhage.

Measurement of cerebral blood flow, arterial blood pressure and intracranial pressure. The techniques used are described in **Supplementary Methods**.

Rabbit tissue collection and processing, laser capture microdissection (LCM), quantitative PCR on rabbit samples and ELISA for PGE2 estimation. The techniques are described in **Supplementary Methods**.

Statistical analysis: Data are presented as mean \pm s.e.m. We compared more than two groups by analysis of variance (ANOVA) for continuous variables and

χ^2 test for categorical variables. We used the Mann-Whitney *U*-test to perform pairwise comparison for continuous variables and the Fisher exact test for categorical variables. A *P*-value of less than 0.05 was considered significant.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

P.B., in whose laboratory this study was performed, planned and supervised experiments, performed confocal microscopy, data analysis and co-wrote the manuscript; H.X. and F.H. performed the animal experiments, immunostaining and western blot analysis; A.B. chose and contributed samples and assisted in editing the manuscript; A.R., K.S., Z.U. and A.C. contributed to laser dissection of brain sections, and to PCR on human and rabbit brains, and also performed the related data analysis; N.L. performed intracranial pressure and cerebral blood flow monitoring of the rabbit pups; S.G. and M.N. assisted in experimental design and planning, and co-wrote the manuscript; M.N. provided direct support for the study.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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